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Effects of fibrinogen degradation products on glomerular mesangial cells in culture

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Effects of fibrinogen degradation products on glomerular mesangial cells in culture. We investigated effects of fibrin, fibrinogen, and fibrinogen degradation products (FDPs) on human glomerular mesangial cells in culture, using the methods of cell count, ^{3}[H] thymidine uptake and ^{51}Cr release. Incorporation of ^{3}[H] thymidine by the cells was much the same with various concentrations of fibrin and similar to findings in the control without fibrin. Fibrinogen, FDP-D, -E, low molecular weight FDP (LMWFDP) fractions 1 and 3 had no promoting effects on mesangial cell proliferation. The LMWFDP fraction 2 showed a suppressive effect on the proliferation of cultured cells and increase of ^{51}Cr release from the cells. The cytotoxic effect of this fraction was dose-dependent. These results suggest that while fibrin deposition in the renal glomeruli does not relate with mesangial hypercellularity, this deposition plays a role in mesangiolysis, through the production of FDPs.

Effets des produits de dégradation du fibrinogène sur des cellules mésangiales glomérulaires en culture. Nous avons étudié les effets de la fibrine, du fibrinogène et des produits de dégradation du fibrinogène (FDPs) sur des cellules mésangiales glomérulaires humaines en culture, en utilisant des méthodes de numération cellulaire, de captation de ^{3}[H] thymidine et de relargage de ^{51}Cr. L'incorporation de ^{3}[H] thymidine par les cellules étaient très voisine avec diverses concentrations de fibrine et identique aux résultats du contrôle sans fibrine. Le fibrinogène, le FDP-D, -E, les fractions 1 et 3 du FDP de faible poids modéculaire (LMWFDP) n'avaient pas d'effets promoteurs de la prolifération cellulaire mésangiale. La fraction 2 du LMWFDP présentait un effet suppresseur sur la prolifération des cellules en culture et sur l'augmentation du relargage du ^{51}Cr à partir de ces cellules. L'effet cytotoxique de cette fraction était dose-dépendant. Ces résultats suggèrent que bien que le dépôt de fibrine dans les glomérules rénaux ne soit pas relié à l'hypercellularité mésangiale, ce dépôt joue un rôle dans la mésangiolyse par l'intermédiaire de la production de FDPs.

Fibrin, fibrinogen, and FDPs have various biological activities. Ishida and Tanaka [1] reported that fibrin had an enhancing effect on the proliferation of smooth muscle cells in culture. Hatzfeld, Hatzfeld, and Maigne described that fibrinogen and its fragment D stimulated proliferation of human hemopoietic cells in vitro [2]. Low molecular weight fibrinogen degradation products (LMWFDPs) have a number of biological activities which include: inhibitory effects on platelet aggregation [3] and blood coagulation [4], chemotaxis for neutrophils [5], enhancement of vascular permeability [6], and cytotoxic effect on cultured endothelial cells [7, 8].

The blood coagulation system contributes significantly to renal damage in various diseases of the kidney. In crescentic glomerulonephritis, fibrin deposition plays a central role in the development of extracapillary proliferation [9, 10]. Fibrinogen-reactive materials may also be found in glomerular tufts under certain conditions, such as acute glomerulonephritis. The pathogenetic significance of this material is unknown.

Krakower and Greenspon [11] isolated renal glomeruli in 1951 and investigations have been done on the biological properties of isolated glomeruli [12, 13] and cultured glomerular cells [14–21]. We attempted to determine the effects of fibrin, fibrinogen, and FDPs on cultured mesangial cells.

Methods

Culture of human glomerular mesangial cells

Fresh kidneys were obtained at autopsy or at the time of resection for surgical treatment of carcinoma of the renal pelvis or hypernephroma. The tissue samples were processed under sterile conditions by a modification of the methods of Foidart et al [22] and Scheinman and Fish [23]. The renal capsules were removed and cortices were dissected away from the medulla. The cortical segments were finely minced into 1 to 2 mm fragments and incubated with 0.2% collagenase (Sigma Chemical Co., St. Louis, Missouri) for 20 min at 37°C. This suspension was then poured through a stainless steel screen of 60-mesh (pore size, 250 μm) and then through a 200-mesh (pore size, 75 μm). The glomeruli and few tubular fragments retained on the 200-mesh were treated with 0.1% trypsin and 0.02% EDTA in phosphate-buffered saline for 10 min at 37°C. After enzymatic digestion, the suspension was pipetted gently to remove Bowman's capsules and visceral epithelial cells, and poured through a 200-mesh on which the glomeruli were retained. The purity of each glomerular preparation was evaluated by phase contrast microscopy and by counting glomeruli. Preparations that contained more than 95% glomeruli were used. Isolated glomeruli were cultured in RPMI 1640 medium (GIBCO, New York, USA) supplemented with 10% fetal calf serum (GIBCO), penicillin 100 U/ml and streptomycin 100 μg/ml. The tissue was transferred to culture dishes (Falcon 3001) and incubated in an atmosphere of humidified air containing 5% CO_2. After about 3 weeks, the cells growing out around

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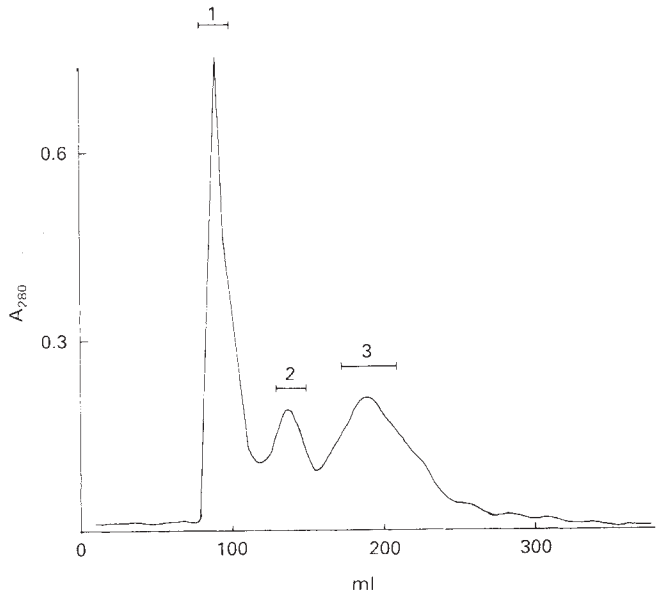

Fig. 1. *Separation of LMWFDPs on Sephadex G-25 column chromatography.* Three fractions (1, 2, and 3) were obtained.

the attached glomeruli were passed through a 200-mesh and subcultured using 0.1% trypsin and 0.02% EDTA. Fourth passages were used in the experiments. For the serum-free control medium, RPMI 1640 medium was supplemented with insulin (NOVO Industry A/S, Denmark) 0.15 U/ml, transferin (Sigma Chemical Co.) 1 μg/ml, selenate Na (Wako, Japan) 1.25 × 10^{-8} M, and monoethanolamine (Wako, Japan) 75 μM.

Purification of fibrinogen, FDP-D, -E, and LMWFDPs

Fibrinogen. Citrated human plasma was absorbed by the addition of solid $BaCl_2$. The Cohn fraction I was obtained by the addition of 50% ethanol to a final concentration of 8% (v/v) at −2°C and 0.055 M Na-citrate buffer pH 6, containing 1 M glycine, 6.5% ethanol [24]. These procedures were repeated three more times. The fibrinogen finally extracted was dissolved in a small amount of 0.01 M phosphate buffer pH 7.0 and lyophilized. Clottability was 96%.

FDP-D and -E. After digesting fibrinogen solution with human urokinase (0.6 U/mg fibrinogen, The Green Cross Co., Tokyo, Japan) for 24 hr, the digests were used for Sephacryl S-300 column chromatography and Pevicon C-870 block electrophoresis [25]. FDP-D and -E fractions were concentrated by PM 10 filter (Amikon, Lexington, Massachusetts) and dialyzed against serum-free RPMI 1640. Identification of these fragments of fibrinogen was made by 7.0% polyacrylamide gel electrophoresis in 0.1% sodium dodecyl sulfate and immunoelectrophoresis analyses using antihuman fibrinogen, antihuman FDP-D, and human FDP-E rabbit sera (Behringwerke AG, Marburg, Germany).

LMWFDPs. Dialysable LMWFDPs were obtained by ultrafiltration of 24-hr urokinase digests of fibrinogen with PM 10. The effusate with a molecular weight of less than 10,000 was chromatographed on a Sephadex G-25 column equilibrated with 0.1 M ammonium acetate pH 7.4 [7]. The first, second, and third peaks were separated and lyophilized (Fig. 1). Protein concentration was determined by the method of Lowry et al [26].

Morphologic confirmation

The glomerular cells were grown on Tissue-Tek chamber slides (Miles, Illinois) fixed with 10% formalin, and stained with toluidine blue. For the demonstration of actin filaments with heavy meromyosin, cultured glomerular cells were washed with phosphate buffer containing 2 mM $MgCl_2$ and 0.1 M NaCl pH 7.0 (buffer A) and treated with 0.1 mg/ml saponin (Sigma Chemical Co.) for 20 min at room temperature in order to permit heavy meromyosin to diffuse into the cells. After washing with buffer A, the cells were treated with 2 mg/ml heavy meromyosin (presented by Professor I. Ohtusuki, Department of Clinical Pharmacology, Kyusha University, Fukuoka, Japan [27]) and 1 mM disopropyl fluorophosphate in buffer A for 6 hr at 2°C and washed with buffer A. These cells were fixed in 3% glutaraldehyde, postfixed in 1% osmic acid, stained with 0.5% uranyl acetate, dehydrated in alcohol, and embedded in Epon. Ultrathin sections were cut, stained with uranyl acetate and lead citrate, and examined with an electron microscope (JEM-100, Japan).

To test fibroblast contamination, the cells were cultured in Eagle's minimum essential medium containing D-valine substituted for L-valine (GIBCO) and dialyzed fetal calf serum [28]. The cultured glomerular cells were tested for their ability to grow in RPMI 1640 containing 50 μg/ml aminonucleoside of puromycin (Sigma Chemical Co.) [29].

Measurement of cell growth

The proliferative effect of fibrin on cultured glomerular cells was examined by uptake of ^{3}[H] thymidine by the cells growing on fibrin-collagen plates. Purified fibrinogen was dissolved in control medium to yield a final concentration of 0.6, 3, and 15 mg/ml; 0.1 ml of fibrinogen solution was poured into tissue culture dishes and 0.2 ml of type IV collagen (10 g/ml, Calbiochem-Behring Co., La Jolla, California) was added. After shaking, 0.05 ml of thrombin (10 U/ml, Mochida Pharmaceutical Co., Tokyo, Japan) was poured into the dishes and agitated gently. The dishes were treated with 0.01 ml of 1 N NaOH and left to stand for 1 hr for gelation of the collagen [30]. One hour later, each dish was washed three times with control medium for 16 hr; 3 × 10^4 cells suspended in control medium were seeded on the fibrin-collagen coated dishes and 10 μl of ^{3}[H] thymidine (0.2 mCi/ml, 5.0 Ci/mmole, RCC Amersham) were immediately added. These dishes were incubated at 37°C in an atmosphere of 95% air and 5% CO_2. Most cells (70% to 80%) attached to the fibrin-collagen coated dishes as well as the collagen coated dishes. After incubation for 1, 2, 4, and 6 days, respectively, triplicated dishes were drained of the medium and the cells were harvested by trypsinization. The acid-soluble fraction was collected and the radioactive DNA was measured with a liquid scintillation counter (Aloka, LSC-903), according to the method of Watson and Yamazaki [31]. Control studies were performed using collagen coated plates and in the absence of fibrin.

The effects of fibrinogen, FDPs, thrombin, and urokinase were examined by direct cell count. Equal numbers (5 × 10^4) were seeded in tissue culture dishes (Falcon 3001) and incubated with control medium containing those agents. After 1,

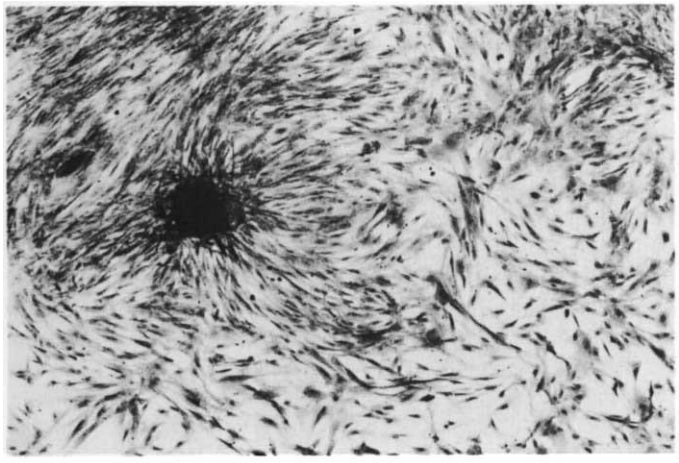

Fig. 2. *Morphology of mesangial cells in fourth passages.* A round nodule, which represents an aggregate of cultured cells, is present. (Toluidine blue, ×25)

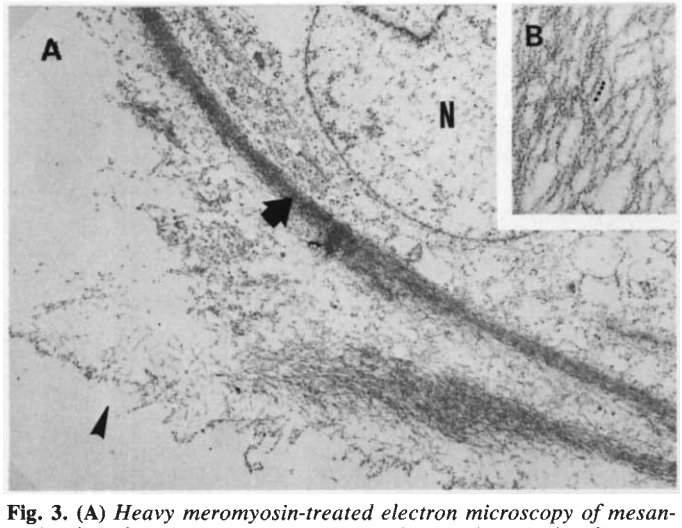

Fig. 3. **(A)** *Heavy meromyosin-treated electron microscopy of mesangial cells in fourth passages.* Abundant thick bundles of microfilaments *(arrow)* are located in the periphery of the cytoplasm. The *arrowhead* refers to the plasma membrane; *N*, nucleus (×4600). **(B)** *An area of A(b) under higher magnification. Arrowhead* appearance is seen along the microfilaments *(series of dots).* (×48,000)

2, 3, 4, 6, and 8 days, the cells were counted in a hemocytometer.

Cytotoxic effects of fibrinogen and FDPs measured by ^{51}Cr release

Confluent glomerular cells cultured in Falcon 3047 dishes (24-well plates) were incubated with NaI $^{51}CrIO_4$ (spec act 3.0 GBq/mg Cr, JRIA) in control medium containing 5 μCi/well for 24 hr. Each well was then washed three times with phosphate buffered saline and 0.5 ml of control medium containing various amounts of fibrinogen and FDP fractions were added. After 24 hr of incubation, 0.3 ml of the medium was collected and the ^{51}Cr radioactivity was assayed in a gamma counter (I). Spontaneous release of ^{51}Cr (II) was measured using the well containing the control medium only, and maximum release of ^{51}Cr (III)

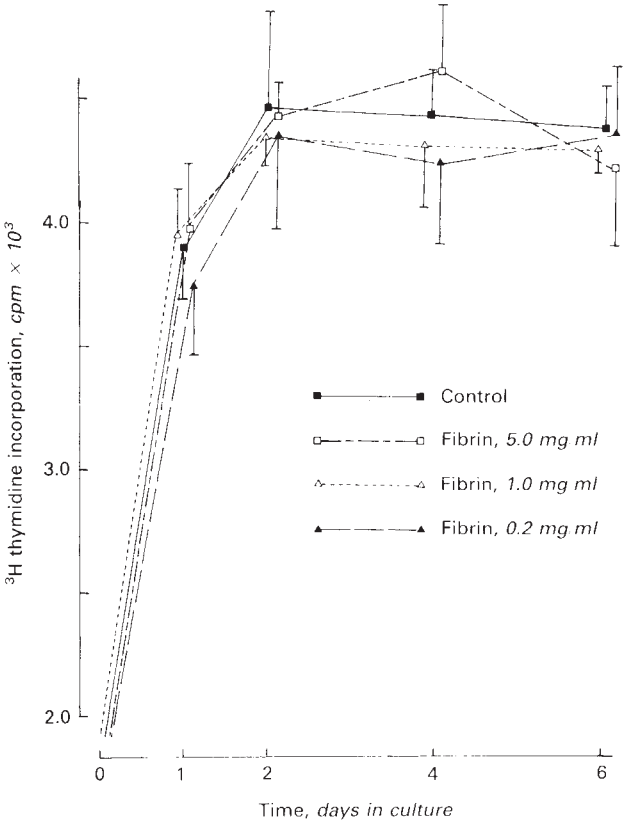

Fig. 4. *Effect of fibrin on the incorporation of ^3H thymidine into glomerular mesangial cells in culture.* Data are the mean ± SEM of triplicate culture.

was measured using the well which had been frozen and thawed three times [7]. ^{51}Cr release (%) was calculated as

$$\frac{I - II}{III - I} \times 100\ (\%)$$

Results

Glomerular mesangial cells in culture

Four or 5 days after initiation of the culture, 15 to 30% of the glomeruli became attached to the bottom of the dish, and we observed the cells creeping out from the glomeruli. Morphologically, the cells appeared homogeneous and were characterized by a stellate or fusiform shape, and by growth in tightly interwoven bundles. Monolayers of large polygonal epithelial cells and a cobblestonelike appearance of endothelial cells were not evident with phase contrast microscopy (Fig. 2).

The mesangial cells contained thick, most often peripherally located, bundles of microfilaments. In the case of heavy meromyosin treatment, these filaments showed an arrowhead appearance characteristic of actin filaments (Fig. 3).

Cell growth was easily maintained in a medium containing D-valine substituted for L-valine, thereby indicating that the cells were not fibroblasts. As the aminonucleoside of puromycin was not cytotoxic toward these cells, the cells were not epithelial tissues.

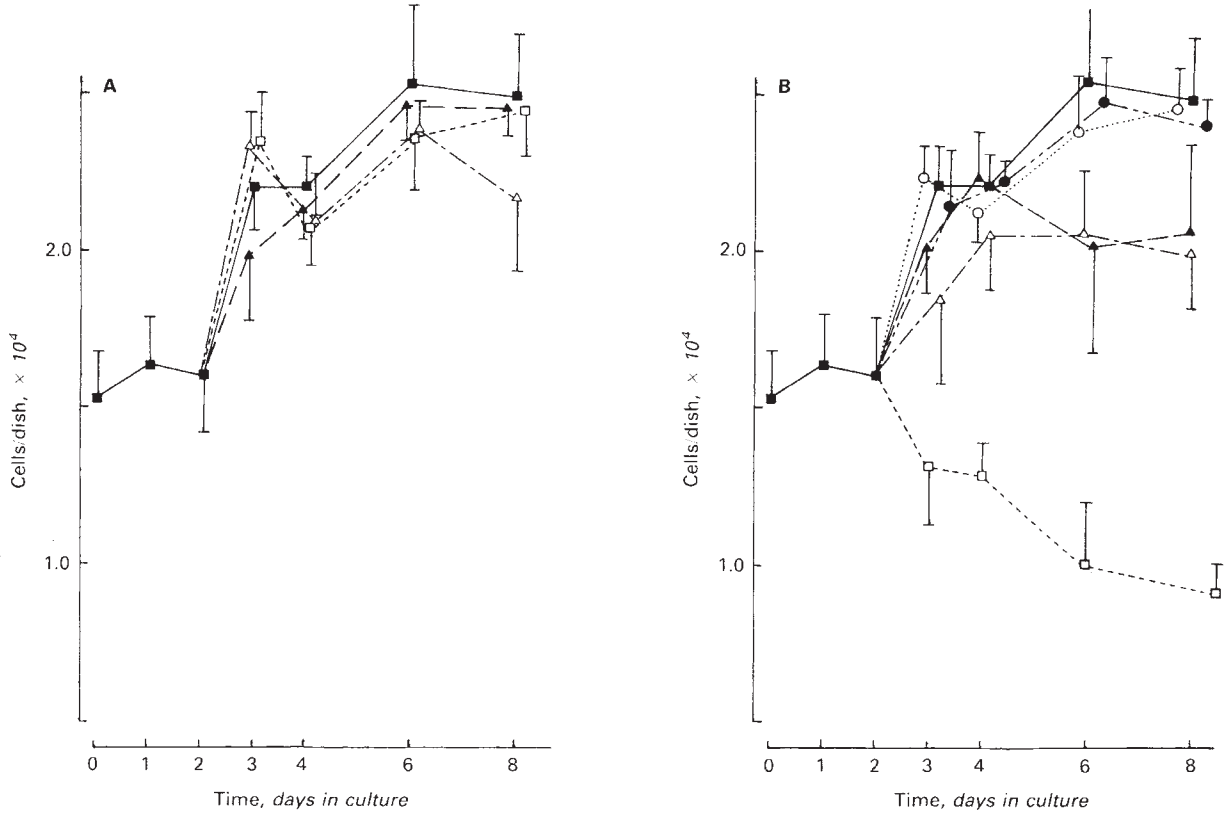

Fig. 5. **(A)** *Effects of fibrinogen* (▲— —▲), FDP-D (□------□), *and FDP-E* (△— - —△) *on the growth of glomerular mesangial cells in culture;* (■——■), the control group. **(B)** *Effects of LMWFDPs, urokinase, and thrombin.* Symbols are: LMWFDP-1 (△— - —△); LMWFDP-2 (□-------□); LMWFDP-3 (▲— —▲); urokinase (●— - —●); thrombin (○· · · · ·○); control (■———■). Triplicated cultures were counted on each experimental day. Each *point* represents the mean ± SEM.

Effects of fibrin on mesangial cell proliferation

Figure 4 showed the growth patterns of mesangial cells proliferating on the collagen plate containing 0, 0.2, 1.0, and 5.0 mg/ml fibrin. Incorporation of ^{3}[H] thymidine by the cells was the same level with various concentrations of fibrin. Enhancing and/or cytotoxic effects were nil.

Effects of fibrinogen, FDPs, thrombin, and urokinase on mesangial cell proliferation

The effects of fibrinogen, FDP-D, and -E added to the control medium were compared with the findings with control media alone. There was no promoting effect on mesangial cell proliferation (Fig. 5A). The effects of LMWFDPs on cell proliferation are shown in Figure 5B. The LMWFDP fraction 2 showed a suppressive effect on cell proliferation as compared to the control media. Other fractions had neither promoting nor suppressive effects on cell growth.

Mesangial cell injuries by fibrinogen and FDPs measured by ^{51}Cr release

Table 1 shows the cytotoxic effects of fibrinogen and FDPs on cultured mesangial cells. Fibrinogen, LMWFDP fraction 1 and fraction 3 showed no significant increase of ^{51}Cr release from mesangial cells. FDP-D and -E had mild cytotoxic properties and the LMWFDP fraction 2 moderate cytotoxic effects in a concentration of 10 μg/ml. The dose dependency of the cytotoxic effects of FDP-D, -E, and LMWFDP fraction 2 is shown in Figure 6. There was an increase in ^{51}Cr release with increasing concentration of the LMWFDP fraction 2. FDP-D and -E had no dose-dependent cytotoxic effects on the mesangial cells in culture.

Table 1. Effects of fibrinogen and FDPs on ^{51}Cr release from glomerular mesangial cells in culture[a]

Maximum release	100 ± 4.7
Spontaneous release	0 ± 0.9
Fibrinogen, *2 mg/ml*	−0.2 ± 0.5
FDP-D, *100 μg/ml*	6.4 ± 1.9[b]
FDP-E, *100 μg/ml*	6.5 ± 0.7[b]
LMWFDP-1, *10 μg/ml*	1.9 ± 3.8
LMWFDP-2, *10 μg/ml*	16.2 ± 5.7[c]
LMWFDP-3, *10 μg/ml*	3.5 ± 2.3
Thrombin, *1 U/ml*	0.5 ± 1.1
Urokinase, *5 U/ml*	4.1 ± 4.3

[a] Each value represents the mean percent ± SEM of six replicate wells.
[b] $P < 0.01$, [c] $P < 0.005$ (compared to spontaneous release).

Discussion

Morphological findings in phase contrast microscopy [32–35] and response to aminonucleoside of puromycin [29] or the medium containing D-valine [28, 29] indicate that cultured glomerular cells are mesangial in origin.

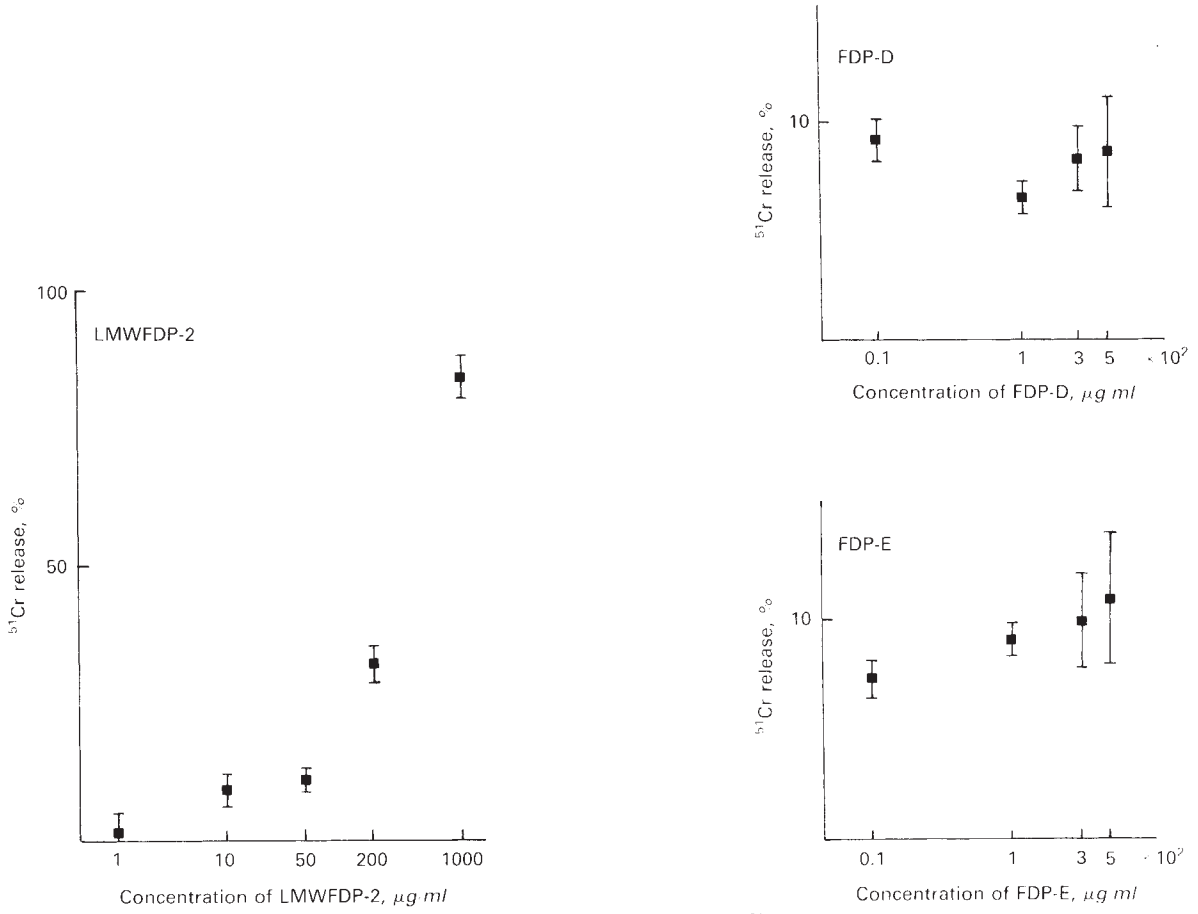

Fig. 6. *Effects of FDP-D, -E, and LMWFDP fraction 2 at the various concentration on ^{51}Cr release from mesangial cells in culture.* Each *point* represents the mean ± SEM of six replicate wells.

Heavy meromyosin specifically binds to actin filaments and a distinctive arrowhead arrangement, visible in the electron microscopy, is formed along the actin filaments [36]. Heavy meromyosin has never been found to bind with microtubules, neurofilaments, intermediate filaments or tonofilaments, thus the binding to a filament is considered to be a distinctive histochemical test for the identification of actin. In our observation, cultured glomerular cells had peripherally located thick bundles of microfilaments and these filaments showed an arrowhead appearance with heavy meromyosin treatment. Therefore, these cells were mesangial in origin.

Fibrin or fibrinogen did not have a promoting effect on proliferation of mesangial cells in culture. In recent studies, the glomerular mesangium was found to contain at least two subpopulations of cells [37, 38]. The first is classical mesangial cells of renal origin and resembling smooth muscle cells, and the second is Ia-antigen bearing monocytelike cells. The Ia-antigen bearing cells are present at the frequency of 1 to 2% of total glomerular cell population in vivo and in the primary culture, and disappear with prolonged culture. In the acute stage of glomerulonephritis, monocytes play an important role in mesangial hypercellularity and removal of deposits [39–41]. Then the possibility, that fibrin deposition is a cause of mesangial hypercellularity through the stimulation of proliferation of Ia-bearing cells and of migration of circulating monocytes, remained unresolved. Although Nakashima et al found that the platelet-derived growth factor stimulated mesangial cell proliferation in culture [42] and Mulcion et al [43] and Wagner, Lucas, and Nagle [44] reported the promoting effect of macrophage products on the proliferation of cultured mesangial cells, other factors, including immune complex or humoral factors released from leukocytes have to be investigated.

Among the FDPs, the LMWFDP fraction 2 had a potent cytotoxic effect on cultured mesangial cells. These results suggest the biological significance of LMWFDP fraction 2 in vivo in the development of the pathologic changes in the glomerular lesions associated with accumulation or deposition of fibrin and fibrinogen. Grond et al described that local hemodynamic factors and deposition of circulating substances such as lipids play a crucial role in the pathogenesis of focal segmental glomerular sclerosis lesions [45]. Velosa et al [46] found that in rats with focal sclerosis induced by aminonucleoside of puromycin, irreversible epithelial cell damage was the initiating event leading to a focal sclerosis and fibrin-related antigen participated in the sclerotic process. All these findings taken together indicate that the LMWFDP fraction 2 inhibits the proliferation of mesangial cells and may disrupt some functional activities, including disposal of deposits, thereby enhancing mesangial sclerosis.

On the other hand, degeneration and necrosis of mesangial

cells lead to mesangiolysis. Mesangiolysis plays a significant role in experimental toxic glomerulopathies [47–49] and various human glomerular lesions [50, 51]. The results of our studies indicate that LMWFDP can be one of the causative agents of necrosis of mesangial cells and mesangiolysis in vivo.

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